Progress in the production of haematopoietic stem and progenitor cells from human pluripotent stem cells

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ABSTRACT

Cell therapies are currently used to treat many haematological diseases. These treatments range from the long-term reconstitution of the entire haematopoietic system using the most potent haematopoietic stem cell (HSC) to the short-term rescue with mature functional end cells such as oxygen-carrying red blood cells and cells of the immune system that can fight infection and repair tissue. Limitations in supply and the risk of transmitting infection has prompted the design of protocols to produce some of these cell types from human pluripotent stem cells (hPSCs). Although it has proven challenging to generate the most potent HSCs directly from hPSCs, significant progress has been made in the development of differentiation protocols that can successfully produce haematopoietic progenitor cells and most of the mature cell lineages. We review the key steps used in the production of haematopoietic stem and progenitor cells (HSPCs) from hPSCs and the cell surface markers and reporter strategies that have been used to define specific transitions. Most studies have largely relied on the use of known markers that define HSPC production *in vivo* but more recently single cell RNA sequencing has allowed a less biased approach to their characterisation. Transcriptional profiling has identified new markers for naïve and committed hPSC-derived HSPC populations and trajectory analyses has provided novel insights into their lineage potential. Direct comparison of *in vitro*- and *in vivo*-derived RNA single cell sequencing datasets has highlights similarities and differences between the two systems and this deeper understanding will be key to the design and the tracking of improved and more efficient differentiation protocols.

1. Cell therapy for haematopoietic disorders and deficiencies

Cell therapy has been used for decades to treat blood and immune cell disorders. Haematopoietic stem cells (HSC) derived from bone marrow, umbilical cord blood or mobilised peripheral blood can reconstitute the entire haematopoietic system in the long term and can be used to treat cancer patients when their haematopoietic system has been ablated by the toxic effects of chemotherapy (1). HSC transplantation also provides a life-long cure for patients with specific genetic disorders that affect the production or function of cells of the blood or immune system. The rapid advances in genetic editing using CRISPR-CAS9 strategies makes gene correction of patient-derived HSCs a realistic option in single

gene disorders including Wiskott-Aldrich syndrome, chronic granulomatous disease and severe combined immunodeficiency in the future (2-4). Current standard practise is to use HSCs derived from healthy donors but incomplete immunological matching can lead to graft-*versus*-host disease (5, 6).

Red blood cell (RBC) loss following trauma or during surgery and genetic disorders including anaemias and thalassamias can be treated in the short term by the transfusion of donor-derived RBCs (7). Platelet transfusion is used to prevent bleeding in people with clotting disorders or low platelet counts of cancer patients receiving chemotherapy (8). More recently there is also increasing interest in the therapeutic application of other mature haematopoietic cells including T cells and natural killer cells as novel cancer treatments and macrophage lineage as a cell therapy for liver fibrosis (9-12).

As stated above, these therapeutic haematopoietic cells are primarily sourced from allogeneic donors but there are limitations in supply and, despite HLA matching of HSC transplants, there remains a requirement for immunosuppression to avoid rejection and graft-versus-host disease (6). Some β-thalassaemia patients require life-long regular blood transfusions that can result in complications including haemolytic transfusion reactions, transfusion-associated graft-*versus*-host disease and circulatory overload (7, 13). The potential to generate these cells from human pluripotent stem cells (hPSCs) could provide a limitless, off-the-shelf source of therapeutic cells that it more amenable to genetic editing compared to primary HSCs and the advent of induced pluripotent stem cell (iPSC) technology could offer an autologous resource (14, 15). Most cells of the haematopoietic hierarchy can be generated *in vitro* from hPSCs but it has become clear that PSC-derived cells are not entirely equivalent to their *in vivo* counterparts (15, 16)(Table 1). For example, it has not been possible to generate long-term reconstituting HSCs *in vitro* without significant genetic intervention and the final enucleation step in the production of fully mature red blood cells does not occur efficiently (17, 18). T lineage cells can be generated but positive selection and the production of mature T cells from human PSCs have been limited (19). The production of functional platelets from iPSC-derived megakaryocytes can be achieved but scaling up of this process to generate enough cells for therapy remains a significant challenge (20). Cells of the monocyte/macrophage lineage can be produced at

scale (21) but it is particularly interesting to note that these are considered to be more akin to myeloid cells generated in the embryo that go on to seed the resident populations within specific tissues rather than adult bone marrow derived cells (22). This is in keeping with the widely accepted idea that PSC differentiation recapitulates embryonic, but not adult haematopoiesis. Functional deficiencies in PSC-derived cells are likely to reflect the progenitor cells from which they originate. Thus, it is critical to consider the strategy for production of haematopoietic stem and progenitor cells (HSPCs) *in vitro*, to fully characterise the HSPCs that can be produced and to compare these to their *in vivo* counterparts. Here we briefly review HSPC production *in vivo* and reflect on the lessons that have been learned and applied to *in vitro* differentiation protocols. We summarise the molecular markers that have been used to define the specific stages and transitions and conclude by describing more recent transcriptional profiling and machine learning that has uncovered novel insights into the process.

2. Production of haematopoietic stem and progenitor cells *in vivo*

The development of HSPCs during mammalian development is complex and the process is precisely coordinated both temporally and spatially (23, 24). First characterised in the mouse embryo, there is increasing evidence demonstrating that the spatial organisation and the chronological timing is comparable during human embryogenesis (25, 26). The first blood cells arise in the extraembryonic yolk sac and include primitive embryonic erythrocytes, megakaryocytes and macrophages (27). This is followed by the appearance of erythromyeloid progenitors (EMPs) from the same location that have the additional potential of production of granulocytes and NK cells (28, 29). The most potent haematopoietic stem cells (HSC) capable of long-term reconstitution first arise in the ventral wall of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region (23, 30) that subsequently colonise and expand in the foetal liver (31). HSCs then migrate to the bone marrow where the lifelong production of adult blood and immune cells is controlled within this specialised haematopoietic niche (32).

3. Production of haematopoietic stem and progenitor cells from pluripotent stem cells *in vitro.*

It is widely accepted that the first two waves of haematopoiesis can be recapitulated *in vitro* from both mouse and human PSCs *in vitro* but it has proven more challenging to identify long-term reconstituting HSCs similar to those derived in the AGM (Table 1) (26, 33, 34). The fact that HSCs can be produced in the human iPSC-derived teratoma setting implies that hPSCs have the intrinsic potential to give rise to this cell type in the appropriate environment (35, 36). The capacity of the microenvironment to unlock the potential of hPSC-derived cells has also been implied by the multilineage long-term reconstitution of genetically modified hemogenic endothelium following injection into the bone marrow of recipient mice (37). There are several reports of other genetic programming strategies have resulted in the successful production of HSCs from hPSCs (37, 38). However, although providing invaluable insights into the mechanisms associated with cell production, teratoma and genetic programming strategies would be really challenging to translate into the clinical setting. Thus, the ultimate goal in the field is to discover well defined and controllable culture conditions that result in the efficient production of fully functional haematopoietic cell types.

Initial studies aiming to produce haematopoietic cells from hPSCs involved the use of serum-containing media and co-culture with supportive stromal cell lines derived from the various haematopoietic tissue including bone marrow, foetal liver and AGM (14, 39, 40). Robust multi-lineage CFU-Cs were generated under such conditions but these first protocols have since been replaced with much more defined serum- and feeder-free protocols involving the stepwise addition of recombinant cytokines and small molecules to activate or inhibit specific signalling pathways (16, 41, 42). One of the first refinements of the differentiation protocols used the knowledge that the haematopoietic lineages are derived from the mesoderm germ layer and that the addition of BMP4 to differentiating mouse ESCs could initiate a process resembling primitive streak formation that proceeds mesoderm induction (41).

3.1. Induction and patterning of mesoderm

Mesoderm forms during early embryogenesis following the ingression of undifferentiated epiblast cells through the primitive streak (PS) with distinct regions of the PS inducing different subpopulations of mesoderm that then goes on to form distinct cell lineages including haematopoietic cells (43). Anterior-posterior positioning in the epiblast and the initiation of gastrulation is controlled by the complex interplay of WNT, Nodal and BMP signalling and the subsequent migration of cells through the primitive streak has been shown to be controlled by FGF and WNT signalling (43-47). Manipulation of all these molecular pathways using cytokines and small molecule agonists and antagonists during the differentiation of PSCs *in vitro* has resulted in the design of differentiation protocols to favour mesoderm rather than endoderm and ectoderm production and to control anterior-posterior patterning (48-53). The fate of nascent primitive streak cells are marked by expression of the transcription factors Mxl1, Brachyury/T and GSC and their fate to anterior endoderm or posterior mesoderm progenitors in differentiating hPSC can be modulated by manipulation of the Activin/Nodal and BMP signaling pathways (51). Initial studies to enhance the production of mesoderm from differentiating hPSCs relied on the quantification of these mesodermal-related proteins or RNA transcripts in dissociated cells (41, 53). However, the production of reporter hPSC lines to track the cell lineages of interest in real time allowed more efficient and high-throughput strategies for protocol testing. For example, the generation of the Mixl1-GFP reporter hESC line provided an important resource to explore germ layer specification of differentiation in hPSC and to show that many of the mesoderm inducing processes, first identified in the mouse embryo, are conserved in human (54). Importantly, the use of this reporter hESC line confirmed the importance of BMP4 signalling in mesoderm differentiation in hESC differentiation (54). Profiling of Mixl1-GFP expressing cells identified additional mesodermal markers including APLNR as cell surface marker for this stage of differentiation (55-57). Mesoderm that differentiates from hPSCs in the presence of BMP4 were considered to be comparable to cells of the posterior primitive streak and express APLNR, KDR (FLK1) and PDGFRα. These cells lack of expression of endothelial (CD31), haematopoietic (eg CD43 or CD45) or endothelial/mesenchymal (CD73, CD105) markers (33) but in the presence of FGF2 and VEGF they differentiate into progenitors with both endothelial and haematopoietic potential that can be tracked by their expression of KDR and their potential to generate blast colonies (BL-CFC) (Figure 1) (58, 59). Signalling pathways involved in the modulation of

mesoderm generation and specification also impact on the balance between the production of cells that resemble extra-embryonic haematopoietic cells (eg primitive erythroid cells and erythromyeloid progenitors (EMPs)) and definitive-like haematopoietic progenitors that arise in the AGM of the embryo (34, 52, 60). For example, the production of primitive haematopoietic cells, marked by expression of CD235a, is temporally dependent on the activin-nodal pathway whereas the production of definitive -like progenitors that express Runx1 is not (52). Simultaneous activation of WNT and inhibition of the TGFβ pathway favours the production of definitive AGM-like haematopoietic progenitors that are also marked by the upregulation of HOXA gene expression (34).

3.2. Endothelial to haematopoietic transition.

Haematopoietic cells arise from specialised haemogenic endothelial cells (HECs) at the various haematopoietic sites during embryonic development in a process known as endothelial to haematopoietic transition (EHT) (24, 26). HECs express the endothelial surface markers VE-cadherin, CD31 and KIT and the transcription factors RUNX1 and GATA2 (16). The process of EHT has been described in differentiating mouse and human PSCs (16, 60, 61). During hPSC differentiation, endothelial cells can be identified using typical endothelial markers such as VE-cadherin (CD144), CD31 and CD34 and the absence of the pan-hematopoietic marker CD43 (33). Although CD34 is commonly used to enrich for adult HSCs from umbilical cord blood or mobilised peripheral blood, it is not specific for HSPCs in differentiating hPSC as it is also expressed on endothelial cells and mesenchymal cells. The finding that the marker CD43 could be used to separate haematopoietic cells from these cell populations represented a significant advance in the field (62).

The heterogeneity of hPSC-derived endothelial cells and the lineage relationship between haemogenic endothelium and haematopoietic progenitors was addressed using a Runx1c-tdTomato reporter hESC line in one of the first single cell transcriptional analyses of differentiating hPSCs (63). The study demonstrated that haematopoietic potential was restricted to the Runx1C-tdTomato^{+} endothelial cells (CD31^{+}CD144^{+}). RNA sequencing confirmed *SOX17* as marker for HE as previously described (60, 64), and identified novel

markers, including *TIMP3*, *ESAM*, *RHOJ*, and *DLL4,* that distinguished haemogenic endothelium from the Runx1C$^-$ endothelium (63).

A more extensive single cell transcriptional analyses of index sorted hPSC-derived cells was carried out to further characterise the EHT process (65). Transcriptional profiling of almost 500 CD34$^+$ cells revealed the heterogeneity of endothelial cells and the presence of transitionary cells that expressed both endothelial and haematopoietic genes. This analysis revealed a continuum of endothelial and haematopoietic signatures with increasing expression of GFI1b correlating with a loss of endothelial gene expression and the subsequent upregulation of other haematopoietic transcription factors such as *TAL1*, *LYL1*, *GATA2* and *RUNX1.* One of the most interesting and somewhat surprising findings from this study was that the endothelial-haematopoietic transitionary population consisted of sub-populations that expressed markers of specific haematopoietic lineage commitment, implying that haematopoietic lineage restriction occurs prior to completion of the EHT process (65).

The emergence of endothelial and haematopoietic lineages in differentiating hESCs was characterised in more detail using a double reporter cell line where mCherry marked the expression of SOX17 in endothelium and GFP marked the RUNX1C isoform in haematopoietic progenitors (34, 60). In these studies, specific culture conditions were employed to mimic either extraembryonic yolk-sac like and intraembryonic AGM-like haematopoiesis. Transcriptional profiling was performed on cells progressing through the different stages of yolk-sac-like haematopoiesis including PDFGRα^+ mesoderm, CD34$^+$SOX17$^-$ and CD34$^+$SOX17$^+$ endothelial cells and the emerging CD34$^+$CD43$^+$ haematopoietic cells. Findings from this analysis supported the concept that SOX17$^-$ endothelial cells differentiate into either a haematopoietic or an endothelial cell fate consistent with the presence of an haemangioblast intermediate whereas acquisition of SOX17 expression was associated with the generation of non-haemogenic endothelial cells (60).

The above single cell transcriptomic studies provided valuable insight into the production of hPSC-derived HSPCs but they depended on the use of known markers for their

identification. Specifically, CD34 and CD43 have been used to define the hPSC-derived HSPCs but, although it is known that CD43 marks the majority of hPSC-derived HSPCS, it is not clear whether this marker is expressed on all HSPCs and specifically on the most potent HSCs capable of long-term reconstitution. Single cell RNA sequencing studies and lineage tracing of haematopoietic cell populations *in vivo* have revealed that HSPCs are far more heterogeneous than first thought, impacting on our characterisation of *in vitro*-generated HSPCs (66) Some cells within the phenotypic HSC compartment expressed transcriptional programs associated with lineage committed progenitors. For example, genes associated with the megakaryocyte lineages including von Willebrand factor, are expressed in a subset of HSCs (67). There is now strong evidence for the existence of lineage-biased HSCs conflicting with the traditional view of the haematopoietic hierarchy (reviewed in (66)).

4. Characterisation of hPSC-derived HSPCs.

To define the heterogeneity of hPSC-derived HSPCs and to assess their lineage trajectory, we designed a minimal membrane marker strategy that allows an unbiased isolation of hPSC-derived HSPCs that were then subjected to single cell RNA-Seq and CITE-Seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing) analyses (68). CITE-Seq strategy adds an additional dimension because it incorporates quantitative information on cell surface marker expression at the protein level. Antibodies against cell surface markers of interest are labelled with oligonucleotides from which antibody-derived tags (ADTs)can be obtained and identify the cells that express that marker in subsequent single cell RNA sequence analyses. It provides a really powerful approach particularly in the haematopoietic system where so many markers are known and it offers the ideal strategy to define the transcriptome of cells expressing novel markers without the need for cell sorting. We used this approach to confirm the expression of a number of novel markers in naïve and lineage committed progenitors that we had identified by clustering and trajectory analyses. For example, we identified and confirmed CD44 as a marker for naïve progenitors which has been since reported by others in single cell sequencing experiment (69) and as a endothelial cell marker in mouse AGM (70). We also identified CD326 (EPCAM) as a marker of haematopoietic progenitors committed to the erythroid lineage and confirmed this finding in erythroid progenitors differentiated from human haematopoietic progenitors derived from umbilical cord blood. ICAM2 and CD9 were identified as markers associated with

megakaryocyte-committed progenitors. Myeloid-committed cells were marked by expression of CD18 but sub-clustering of the transcriptional dataset revealed that this $CD18^{+}$ population could be subdivided into progenitors with eosinophil, neutrophil, and monocyte potential (71).

In addition to the expected markers such as CD34, CD44 and CD33 we also identified CD132, CD52, CD180 and IL3RA as potential candidate markers for the most potent HSC-like cells and propose these could be used both for their isolation and track their production. Gene network analyses identified LMO4 as a potential regulatory transcription factor that had not been previously associated with HSPCs. Interestingly we also identified ID2 and ID4 in naïve progenitors that, as targets of BMP signalling, could provide one of the mechanisms associated with the important role of this pathway in HSPC production (71).

5. The haematopoietic hierarchy.

Although it has provided an excellent framework for experimental haematology, there is increasing evidence against the classical hierarchical model of haematopoiesis where mature cells of the blood are generated via multipotent, oligopotent then unipotent progenitor cells (72-74). The use of additional markers to subset common myeloid progenitors (CMP) and improved assays for function readouts (73) as well as impressive single cell transplantation assays (72) have demonstrated that myeloid cells can be generated directly from HSC without progressing through an intermediate CMP. There is also strong evidence for the existence of HSCs that are biased towards the production of platelets that could be activated in acute emergency situations such as life-threatening platelet depletions associated with acute inflammation (75). Single cell transcription analyses support the idea of a continuum of differentiation states rather than discrete cell stages (74, 76-78).

Trajectory analyses of hPSC-derived HSPCs also demonstrated a continuum of cell states to specific cell lineages as opposed to a sequential hierarchy of discrete cell types (71). We identified a central cluster of naïve progenitors that expressed known markers of multipotent HSPCs, such as *GATA2*, and a number of trajectory branches comprising

continua of cells each expressing genes associated with specific lineages. An erythroid branch marked by the expression of *KLF1* and *MYC*, as well as a megakaryocyte branch by expression of *GATA1, TAL1* and *FLI1* were identified. Granulocyte-committed cells were represented by a separate branch and demonstrated the expression of *CEBPD, CEBP-B, CEBP-A* and *CEBP-E*.

6. Comparison of *in vitro* and *in vivo*-derived HSPCs.

CD43 has been widely used as a marker for hPSC-derived HSPCs but we noted that, although the majority of suspension cells arising from the adherent endothelium expressed this marker, there was a small proportion of RUNX1C-GFP$^+$ cells that were CD43-negative (71). Intrigued by this finding, we carried out one single cell RNA sequencing experiments on CD43$^+$ selected cells and another that captured both CD43$^+$ and CD43$^-$ cells. We then compared the transcriptional profile of these populations with *in vivo*-generated cells using machine learning. We first trained an artificial neural network (ANN) on a scRNA-seq dataset derived from human fetal liver (78) then used that to 'recognise' the comparable cell types that were generated *in vitro* from hPSCs. A range of HSPC phenotypes were identified including a rare population that could be classified as HSCs. This HSC-like population was transient, present in the suspension cells at day 10 but decreased in number when cells were collected later at day 13. Most interestingly, this population was completely absent from the dataset that had been generated using cells selected on the basis of CD43 expression (71). This implies that the most immature human HSCs might not express CD43, a phenotype that is comparable to mouse Pro-HSCs prior to their maturation into functional definitive HSCs (79), and that the culture conditions employed are not conducive to their maintenance and/or maturation.

By comparing the single cell transcriptome of *in vitro*-generated HSC-like cells with those colonising the foetal liver (78), we identified transcription factors and molecular pathways that can be targeted in the aim of improving HSC differentiation *in vitro*. For example, *EGR1* and other members of the early response genes family (*ZFP36L1, NR4A1, FOS, JUN,* and *JUNB*) were expressed at a lower level in the *in vitro*–produced HSC-like cells, and we propose these could be useful in direct programming strategies for HSC production.

It is important to note that some of the transcriptional differences between *in vivo* and *in vitro* derived HSPCs possibly reflect *in vitro* culture conditions rather than intrinsic differences. For example, we detected differences in the expression of genes associated with oxidative phosphorylation indicating metabolically differences (71) which is not surprising considering that oxygen levels in normoxic culture are likely to be significantly higher than in the reported hypoxic environment in the AGM and foetal liver (71, 80). We also reported higher expression of genes associated with the cell cycle in the *in vitro* HSPCs compared to those of the foetal liver. The quiescent nature of the most potent HSCs is well documented with exit from the quiescent state being a critical step in the production of HSCs (81, 82). Given the cocktail of cytokines required for the complex *in vitro* PSC differentiation it is unlikely that that quiescent state is replicated in these conditions. However, the recent characterisation of HSC in hibernation conditions at the single cell level will hopefully provide insight into this critical step during HSC production (65).

7. Summary

The production of fully function therapeutic haematopoietic cell types from hPSCs has enormous potential for the future treatment of disease but success in their production *in vitro* is dependent on our ability to recapitulate the paths involved in their development and maintenance *in vivo*. Here we review the key steps and transitional events in embryonic haematopoiesis that have been considered in the design of differentiation strategies as well as the marker strategies used to monitor the production of HSPCs from hPSCs. The design and implementation of improved protocols is expected to follow from the transcriptional comparison of *in vitro* and *in vivo* generated cells (71). It is evident that the use of precisely defined culture conditions have some limitations and so future strategies may well return to the use of stromal cells and organoids in 3D perfusion bioreactors to mimic the complex conditions required for the production and maintenance of HSCs (83). It is now well established that hemodynamic forces are required for HSPC formation *in vivo* and so the application of sheer stress to differentiating hPSCs or the activation of genetic pathways that are activated by mechanical forces may provide one of the vital steps in the production of functional HSCs from hPSCs (84).

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9. References

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10. Figure Legends

Table 1

Characteristics of human PSC-derived haematopoietic cells compared to their counterparts generated in vivo.

Figure 1

Differentiation of human pluripotent stem cells into haematopoietic cells highlighting the signalling pathways that have been modulated to control the key transitionary steps and the molecular markers that have been used to track the process.